Contributions to the Studies on the Pathogenicity and Virulence of the Tubercle Bacillus : Hemolysis caused by the dense bacterial suspension

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Hemolysis caused by the dense bacterial suspension

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Many interesting studies have recently been made on the relationship between the virulence of tubercle bacilli and their morphological, biological and cytochemical characters, namely those of Middlebrook and Dubos,[1] Allgöwer and Bloch,[2] Dubos[3] and others. In succession to these studies, some peculiar behaviors of tubercle bacilli being considered to relate to their virulence are studied by the present writer. The observation of a kind of hemolysis caused by the dense bacillary suspension of tubercle bacilli is stated in the following.

Materials and methods

The almost all of the test-bacilli were stock culture strains in our laboratory and the others were those isolated recently from patients or soils.

Human type	
Frankfurt	virulent
H37Rv	virulent
Asakura	virulent
Aoyama	attenuated
T_2 T_5, T_6	virulent, isolated recently from sputum of patients
Bovine type	
Bl	virulent
Rm	virulent

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B15	virulent
BCG	remarkably attenuated
Vole Bacillus	
Avian type	
Choju	virulent
Cho 71	virulent
Chokyo	attenuated
Non-pathogenic mycobacteria	
Smegma bacillus	
Other six strains	isolated from soils by Söhngen's method[1]

The bacilli growing on the surface of Sauton's synthetic medium were taken and washed with distilled water in order to remove the ingredients of culture media, were suspended somewhat densely in distilled water. A few drops (about 0.05 ml) of the dense suspension were smeared making a circle about 5 mm in diameter with a pipette and dried aseptically. Slices of paper (0.05mm in thickness) were put near both lateral edges of the slide. Then blood was taken out from test animals and dropped in small dosage from a syringe on the dried bacterial smear. Another sterile slide was then carefully placed on it. The edges of the slides were sealed with melted paraffin when it was certain that the blood between two slides has already been coagulated. The slides thus prepared and kept horizontally were incubated at 37°C and the results were studied after 24 hours.

This hemolysis was only observed in the part of coagulated blood which corresponded to the width of bacterial smear. It was, therefore, possible to doubt the contamination if this phenomenon occured beyond widely surpassing the width of bacterial smear. As the width of the bacterial smear was not always constant, the thickness of the dried bacterial layer had some differences in each case. From this reason, the observation was made always with more than three specimens under the same condition.

Results

1. Examinations of the conditions necessary for the appearance of the hemolysis

a) Density of bacillary suspension and the hemolysis.

To know the density of bacillary suspension necessary to produce the hemolysis, human strains (Frankfurt and H37Rv) were tested with the blood of guinea pigs. The hemolysis was not brought about with low concentrations of bacillary suspension. The concentration more than 50 mg/ml was necessary for the appearance of the complete hemolysis (Table 1).

From these results, it may be probable that this phenomenon has been

overlooked by other workers as this is produced only with dense bacillary suspension.

Table 1

Density of the bacillary suspension and the hemolysis.

Density of the bacillary suspension	1 mg/ml#	5 〃	10 〃	20 〃	50 〃	100
H37Rv	− − −	− − −	− − −	− − ±	+ + +	+ + +
Frankfurt	− − −	− − −	− − −	− ± ±	+ + +	+ + +

\+ complete hemolysis……transparent in the entire part corresponding to the bacterial smear.
± incomplete hemolysis……transparent only in the part corresponding to the marginal part of the bacterial smear.
− none of hemolysis……not transparent.
\# wet weight

b) The time and temperature of the reaction and hemolysis.

The time and temperature of the reaction necessary to produce the hemolysis were examined with the blood of guinea pigs using the suspension of 50mg/ml of the human strain (Frankfurt) (Table 2). The optimal temperature of the reaction was 37°C; and in room temperatures at 10°C and thereabout, the hemolysis occured later and less intensely. In an ice-box, the hemolysis was not seen but it was observed when the same specimen was taken out from the ice-box and preserved at 37°C.

Table 2

The time and temperature of the reaction and the hemolysis

Time (hrs.)	12	24	48	72
Ice box	− − −	− − −	− − −	− − −
Room temperature	− − −	− − −	− − −	− ± ±
37°C	− − −	+ + +	+ + +	+ + +

Strain: Frankfult 50 mg/ml

It was not reasonable to keep the specimen higher than 37°C, as spontaneous hemolysis occured in almost entire part of the coagulated blood. The time appropriate to the reaction was 16 to 24 hours at 37°C. Before 12 hours the hemolysis did not occur completely. On the other hand, later than 24 hours the hemolysis frequently was provoked by the contamination.

c) Individual difference of the bloods of guinea pigs and hemolysis.

In order to examine whether the bloods offered by different individums of guinea pigs show some differences in this reaction, the following observation was made with the human strains (Frankfurt and H37Rv). As seen in Table 3, there was almost no differences among the bloods offered by six guinea pigs employed.

Table 3

Individual differences of the bloods of guinea pigs and the hemolysis.

Guinea pigs	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Human Strain						
Frankfurt	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
H37Rv	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +

Bacillary suspension……50 mg/ml

d) Culture ages of tubercle bacilli and the hemolysis.

Table 4

Culture ages and the hemolysis.

Culture ages (weeks)	1	4	10
Strain Frankfurt	± + +	+ + +	+ + +
Strain H37Rv	± ± +	+ + +	+ + +

Blood of Guinea pigs was used.

As seen in Table 4, though there was no remarkable difference following culture ages, the tendency was observed that the older the culture the stronger the hemolysis.

e) The hemolysis with living and dead bacilli and culture filtrates.

It is a well-known fact that the ratio of living cells of tubercle bacilli contained in the surface cultures varies following the culture ages. The culture ages had no particular influence upon the degree of hemolysis as seen in Table 4. From this datum, it seemed to exert no influence on the hemolysis whether the bacilli were living or dead.

Examining with heat-killed bacilli, one could bring about almost as much hemolysis as that with living ones of the same concentration (Table 5). The water soluble constituent of tubercle bacilli extracted by grinding in a mortar with water produced no hemolysis. Also, hemolysis was provoked neither with the culture filtrate (Seitz) of glycerol broth of the human strain (Frankfurt) for 4 weeks, nor with the tuberculin diluted ten times with distilled water.

Table 5

The hemolysis with living and dead bacilli and culture filtrates.

	Living bacilli 50mg/ml	Dead bacilli 50mg/ml	Water soluble constituent of ground bacilli	Culture filtrate	# Tuberculin
Hemolysis	+ + +	+ + +	+ + +	− − −	− − −

Bacteria: Strain Frankfurt

Blood: Guinea pigs

Product of the Institute for Infectious Diseases, Tokyo University.

2. Pathogenicity and Virulence of tubercle bacilli and the hemolysis.

a) Relationship between the virulence and hemolysis.

The relationship between the virulence and hemolysis was studied with the cultures of 7 human, 4 bovine, 3 avian, 1 vole and 7 non-pathogenic mycobacteria (Table 6).

Among 7 human strains grown on Sauton's media for six week, 4 stock cultures produced hemolysis of different intensity. The hemolysis was the most outstanding with Frankfurt strain, and rather weak with Aoyama strain

Table 6

Virulence and the hemolysis

Types	Strains	20 mg/ml			50 mg/ml			100 mg/ml		
Human	Frankfurt	±	±	+	+	+	+	+	+	+
	Aoyama B	−	−	−	+	+	+	+	+	+
	H37Rv	±	±	±	+	+	+	+	+	+
	Asakura	±	±	±	+	+	+	+	+	+
	T_2	±	±	+	+	+	+	+	+	+
	T_5	−	−	±	+	+	+	+	+	+
	T_6	−	+	±	+	+	+	+	+	+
Bovine	B 1	+	+	+	+	+	+	+	+	+
	Rm	−	−	±	+	+	+	+	+	+
	B 15	−	−	±	±	+	+	+	+	+
	B C G	−	−	−	−	−	±	±	+	+
Vole	Vole	−	−	−	−	±	±	+	+	+
Avian	Chokyo	−	−	−	−	±	±	+	+	+
	Choju	−	−	±	+	+	+	+	+	+
	Cho 71	−	−	−	±	±	+	+	+	+
Non-pathogenic myco-bacteria	Smegma	−	−	−	−	−	−	−	−	−
	F 5	−	−	−	−	−	−	+	+	+
	A 1	−	−	−	−	±	±	+	+	+
	F 4	−	−	−	−	−	−	−	−	−
	E 5	−	−	−	−	−	−	−	−	−
	Fujii	−	−	−	−	±	±	+	+	+
	Kosugi	−	−	−	−	−	−	±	±	+

which had more or less attenuated virulence. H37Rv and Asakura strains showed the intermediate activity between these two strains described above. Three virulent strains, isolated recently from the sputum of patients showed nearly as much hemolysis as that with H37Rv. It may be noticeable that the human strains employed brought about the incomplete hemolysis with 20 mg/ml and the complete hemolysis with 50 mg/ml, though a little difference in hemolysis may exist in each strains.

Among 4 bovine strains, Rm had the highest virulence against guinea pigs but produced somewhat weaker hemolysis than B 1 strain which had more or less attenuated virulence, but had the most active influence upon the guinea pigs blood. B 15 strain was inferior to Rm and B 1 strains, both in the virulence and hemolysis. BCG strain was far less active in hemolytic than these three bovine strains mentioned above, having almost no hemolysis action even with 50 mg/ml. From this result, it is apparent that there are distinct differences between these virulent strains and BCG.

Vole bacillus showed only the similar activity as that of BCG in hemolysis and virulence.

Among three avian strains cultivated for 4 weeks, Choju strain was the most virulent and brought about the most intense hemolysis, Chokyo and Cho 71 strains were less hemolytic.

Smegma bacillus and other six strains of non-pathogenic mycobacteria isolated from soils showed little or none of activity. Even with the high concentration of the bacillary suspension, none or only weak hemolysis occured.

It is sure, from this observation, that this hemolytic reaction was distinct in virulent strain and less intense in BCG and Vole bacillus and rather weak in non-pathogenic mycobacteria.

It may be certain that the hemolytic activity of each strain ran almost parallel with its activity.

b) Relationship between the bloods of various kinds of animals and hemolysis.

The bloods of human, goat, rabbit, guinea pig and cock were examined using human bacillus (Frankfurt) and avian bacillus (Choju) as the test organisms (Table 7).

Table 7

Hemolysis in the bloods of various kinds of animals.

Types	Bloods of Animais	20 mg/ml	50 〃	100 〃
Human (Frankfurt)	Human	− − −	− − −	± + +
	Goat	− − −	− − −	+ + +
	Rabbit	− − −	+ + +	+ + +
	Guinea pig	± ± +	+ + +	+ + +
	Cock	− − −	− − −	± + +
Avian (Choju)	Human	− − −	− − −	± + +
	Goat	− − −	− − −	± + +
	Rabbit	− − +	+ + +	+ + +
	Guinea pig	− − −	− − −	+ + +
	Cock	− − −	− − −	+ + +

The hemolytic action of the human bacillus (Frnkfurt) was the most remarkable in the blood of guinea pig, and less remarkable in those of rabbit, goat, human and coak, while the avian bacillus (Choju) was the most active in the blood of rabbit, and less active in that of cock.

From this observation it may be said that there is no specific relationship between the blood of various kinds of animals and the types of bacilli employed.

Referring to the above-mentioned result, the resistance of the erythrocytes of various kinds of animals to the saline solutions of different concentrations was examined. As seen in Table 8, it may be reasonable to consider that the hemolysis provoked with tubercle bacilli was different from that in saline solution of lower concentrations.

Table 8

Resistance of erythrocytes of various kinds of animals.

NaCl %	8.5	8.0	7.5	7.0	6.5	6.0	5.5	5.0	4.5	4.0	0
Human	−	−	−	−	−	−	+	+	+	+	+
Guinea pig	−	−	−	−	−	−	−	−	−	+	+
Goat	−	−	±	+	+	+	+	+	+	+	+
Rabbit	−	−	−	−	−	±	±	+	+	+	+

\+ complete hemolysis
± incomplete hemolysis

c) The bloods of tuberculous guinea pigs.

In order to examine whether some immunological factors like hemolysin take part in this hemolysis, observations were made with the bloods of normal and tuberculous guinea pigs. As shown in Table 9, there was no difference

Table 9

The bloods of tuberculous guinea pigs.

Strain: Frakfurt		10 mg/ml			20 〃			50 〃			100 〃		
Normal guinea pig		−	−	−	−	±	±	+	+	+	+	+	+
Tuberculous guinea pigs	No. 1	−	−	−	−	−	±	+	+	+	+	+	+
	No. 2	−	−	−	±	±	±	+	+	+	+	+	+
	No. 3	−	−	−	−	±	±	+	+	+	+	+	+

\+ Complete hemolysis
± imcomplete hemolysis

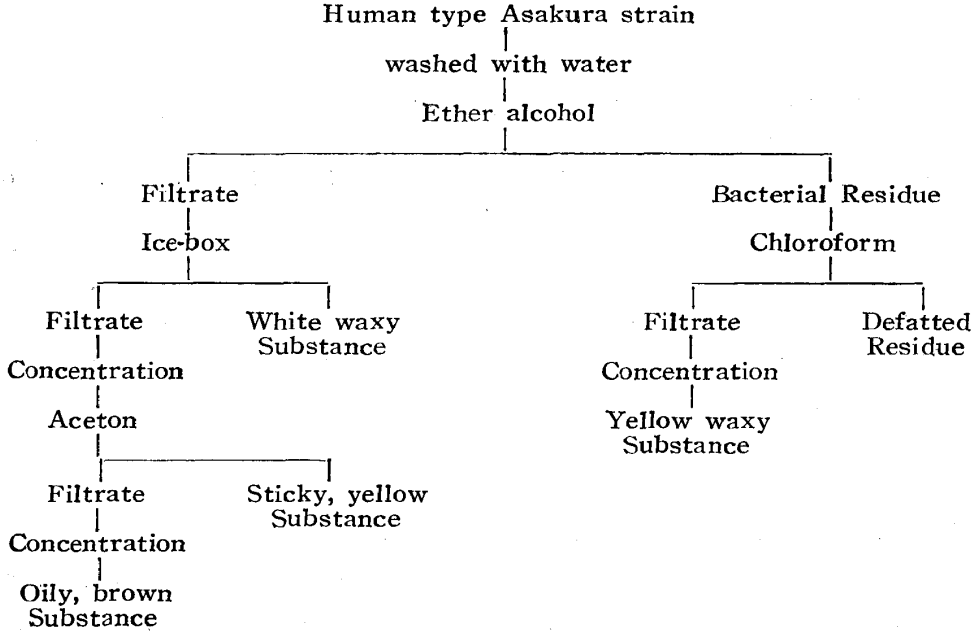
between the blood of normal guinea pigs and that of tuberculous ones. In other words, it may be said that this hemolysis is probably provoked without any correlation with tuberculous immunity or allergy.

3. Isolation of the constitutents of tubercle bacilli responsible to this hemolysis.

It is already sure that the active substances contained in tubercle bacilli is not such an agent as hemolysin. This hemolytic reaction produced only with bacilli themselves regardless of their vitality, and not with culture filtrate or tuberculin. The hemolytic substances may therefore be some of the bacillary constituents, above all, some of lipid fractions insoluble in water.

The isolation of the hemolytic substances was, therefore, tried by means of the method somewhat modified of Anderson's method[5] for lipid analysis (Table 10).

Table 10
Isolation of hemolytic substances of tubercle bacilli.



The human bacillus (Strain Asakura) grown on Sauton medium was washed thoroughly with water and put into the mixture of alcohol and ether of equal quantity, and extracted at the room temperature. The extracted liquid was kept in an ice-box for 48 hours which produced white waxy sediments. After the sediment was removed, the supernatant was concentrated and added with acetone and then divided into insoluble sticky substance (phosphatide) and acetone-soluble fraction. This procedure was repeated by dissolving the former in ether and precipitating by adding acetone. The acetone-soluble fraction was concentrated to acetone soluble fatt which was oily and brown in color. Bacterial residues were added with chloroform and separated into two parts: one was yellow waxy substance soluble in chloroform and the other was defatted bacterial residue.

With these five fractions, the hemolysis was examined using the blood of guinea pigs. The most intense hemolysis was recognized using the oily brown substance (acetone-soluble fatt). The hemolysis appeared meanwhile at 37°C,

namely from 30 minutes to 2 hours, while in the case of tubercle bacilli themselves it took at least 15 hours as mentioned above. Moreover, the reaction in this case was very powerful, and one could remark the hemolysis spred far beyond the part of the coagulated blood corresponding to the bacillary smear. The sticky yellow substance (phosphatide) produced the hemolysis, but less remarkable than the former. No such reaction was recognized in white waxy substance, chloroform-soluble fraction and defatted residues.

In this way, the active substances contained in tubercle bacilli and responsible to this hemolysis may possibly be lipid which is assumed as acetone-soluble fatt and phosphatide-like substances.

Discussion

It may be the reason why this hemolysis phenomenon has been overlooked by other workers that large amount of bacilli is indispensable to produce the reaction and at the same time the active substances contained within bacilli are insoluble in water. The method applied by the present author resembles somewnat the slide cell culture of Wright. As in the slide cell culture, however, the minute dose of bacilli was used to mix with the blood, it might be possible that one could not observe in this case the hemolysis.

The optimal temperature to induce this reaction was about 37°C, and such a reaction did not arise in an ice-box. It may be because of the insolubility at low temperature of the lipids being responsible to this reaction.

This hemolytic substance is contained in the acetone-soluble fatt and phosphatide fraction as mentioned above. According to Sabin,[6] these fractions contain phthioic acid and tuberculostearic acid being considered as the specific constitutents of tubercle bacilli. It may be interesting to study whether the Sabin's substance and this hemolytic substance are the same or not.

It may be possible, from the above mentioned results, to conclude that this hemolytic reaction owes its outbreak to the reduced surface tension, but neither to the immunological factor nor osmotic pressure.

The results obtained above that the older cultures were generally more active in this hemolysis than younger ones are suggestive if one examine the recent report of Asselineau[7] who confirmed that fatty acid contained in reduced quantity within the cultures at early stages increased following the culture ages.

From the experiment it may be possible to say that the hemolytic action of tubercle bacilli runs parallel with their virulence. Therefofe, it is easy to distinguish non-pathogenic mycobacteria from tubercle bacilli and also it is possible to differentiate BCG and Vole bacillus from virulent strains.

In addition to these considerations it may also be allowed to presume that this hemolytic substance could play some role in the pathogenesis of tuberculosis.

Summary

The hemolytic action of the tubercle bacillus was studied as one of its biological properties.

The active substances of this hemolysis were contained in the acetone-soluble fatt and phosphatide, namely in lipid fraction of tubercle bacilli.

This hemolysis made possible to distinguish in vitro the pathogenic mycobacteria from the non-pathogenic ones and also to differentiate attenuated bacillus from virulent ones.

This hemolytic action may be considered as one of the main factors on which the virulence of the tubercle bacillus possibly depends.

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